Title: Ecology of Arboviruses in Thailand

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Objectives

a. To determine the ecologic factors which affect the maintenance and dissemination of arboviruses causing human disease in Thailand.

b. To develop laboratory techniques for support of field investigations.

Description

Laboratory studies designed to compare the vector potential of Aedes aegypti and Aedes albopictus for dengue viruses have been initiated. Initial experiments were carried out to compare the infectivity of dengue—2 virus and measure rates of virus growth in both Aedes species. Viremic gibbons were used as a source of infected blood.

An observation was made in this laboratory that suspensions of normal mosquitoes inhibit the infection of cell cultures and suckling mice by certain arboviruses. The virus—inhibiting properties of suspensions of A. aegypti mosquitoes for dengue and chikungunya viruses, and of Culex tritaeniorhynchus and Culex gelidus for Japanese encephalitis viruses were investigated.

In an effort to ascertain relative ecologic isolation of JE virus in various geographic areas of Asia, antigenic analysis of several strains was carried out. Virus strains from Japan, Taiwan, Thailand, India and Sarawak were compared, homologous and heterologous plaque reduction neutralization tests using single—immunization antisera were used.

Studies to determine the natural history of Japanese encephalitis virus in an endemic study area at Bang Phra have continued. Attempts were made to identify reservoir species by continuous serological monitoring of free—living vertebrates to detect the presence of neutralizing antibody. In these "suspect" species in which neutralizing substances were found, their potential role in virus maintenance was determined by infection—viremia experiments. A serologic survey was conducted to determine age—specific neutralizing JE virus prevalence in school children resident in the Bang Phra area. Studies on the known mosquito vectors, C. tritaeniorhynchus and C. gelidus were continued. Collections of mosquitoes attracted to "suspect" vertebrate species were made for comparison of species and numbers of individuals collected by other methods and to test for presence of viruses in them.

Virologic studies at Bang Phra have shown that several other viruses are associated with mosquitoes in the area. Newly recovered agents have been studied in detail to establish their identity and, with Batai and Wesselsbron viruses, serologic studies have been done with the wild vertebrate and human sera from the study area.

Progress

Comparative Infectivity of Dengue-2 Virus for Aedes aegypti and Aedes albopictus

To evaluate the relative vector potential of A. aegypti and A. albopictus attempts were made (1) to infect each species with varying virus doses to compare susceptibility and (2) to determine the relative rates of virus growth in the mosquitoes and the time required for detectable amounts of virus to accumulate in the salivary glands. To simulate natural conditions groups of mosquitoes were fed on gibbons which had been inoculated with 1000 plaque-forming units of dengue-2 virus (BKM-1749, LLC-MK_2 $passage_1$)

Mosquitoes of both species ingested blood containing various quantities of virus. This was done by taking advantage of day-to-day and gibbon-to-gibbon variation in viremia; mosquitoes were fed daily on gibbon S70 through the 14th postinoculation day and on gibbons S8, S9 and S27 on days 3, 5 and 7 after injection. Blood specimens were drawn from the gibbons every day; the serum was separated from the chilled blood and frozen at -70°C and serum virus content measured by LLC-MK_2 cell plaque assay. Engorged mosquitoes were held from 7-11 days at 30-33°C daily fluctuating temperatures and relative humidity of 85-100%. At the end of the holding period, salivary glands were dissected from 1-5 specimens per group and fixed in acetone for the detection of virus by the fluorescent antibody (FA) technique. The remaining mosquitoes were frozen in individual containers until triturated and assayed individually for virus presence by the direct and delayed LLC-MK_2 cell plaque system. The presence of nonspecific inhibitory substances in the gibbon sera made precise viremia quantification by direct plaque assay unreliable. These sera are currently being reassayed in dilution by the delayed plaqe system.

Table 31. Comparative infection of A. aegypti and A. albopictus 7—11 days after feeding on gibbons experimentally infected with dengue type—2 virus.

			Infection Rates	
	Gibbon	Viremia[2]/	A. aegypti	A. albopictus
Day[1]/	No.	Titer	Pos/Total (%)	Pos/Total (%)
1	S70	neg	2/7 (29)	4/11 (36)
2	S70	$10^{0.3}$	3/7 (43)	7/12 (58)
3	S8	$10^{1.3}$	3/5 (60)	2/4 (50)
	S9	neg	1/4 (25)	2/4 (50)
	S27	$10^{1.3}$	0/10 (0)	2/11 (18)
	S70	$10^{1.3}$	3/6 (50)	3/11 (27)
5	S8	neg	1/6 (17)	1/4 (25)
	S9	$10^{1.3}$	2/6 (33)	6/16 (37)
	S27	$10^{0.3}$	3/5 (60)	5/5 (100)
6	S70	$10^{0.3}$	4/6 (67)	9/11 (82)
7	S8	neg	3/7 (43)	4/7 (57)
	S9	$10^{0.3}$	7/9 (78)	2/2 (50)
	S27	$10^{0.3}$	0/2 (0)	1/9 (11)
	S70	$10^{2.3}$	4/5 (80)	7/9 (78)
8	S70	$10^{0.3}$	0/12 (0)	0/5 (0)
9	S70	neg	0/7 (0)	0/13 (0)

1/ Day after inoculation of gibbons.
2/ Highest dilution of serum in which virus detected per 0.3 ml.

Results to date indicate that both species are highly susceptible to infection by dengue-2 virus under these experimental conditions. Both species were infected with equal ease despite very low detectable viremias in the gibbons upon which they fed (table 31). Daily assays of individual mosquitoes of both species for virus presence by plaque and FA through the 7th day following engorgement of gibbon S70 indicated

that the virus multiplies in both species at about the same rate (fig. 11). The mosquitoes ingested approximately 3 pfu of virus. By 24 hours, no infectious virus was detected. Thereafter, the amount of virus in the infected mosquitoes increased rapidly from days 2-6, at which time (days 6-7) it became detectable in the salivary glands by FA.

Arbovirus Inhibitory Substances in Suspensions of Normal Mosquitoes

Implicit in commonly used methods for recovery of arboviruses from mosquitoes is the assumption that no inhibitory substances are present in mosquito suspensions which might prevent infection of cells or mice by the viruses. In experiments with suspensions of newly emerged, unengorged female mosquitoes, infections by arboviruses of LLC-MK_2 cell cultures and, to a much lesser degree, suckling mice, were inhibited.

Mosquito suspensions were made by the same basic procedures used in this laboratory for virus isolation attempts. Twenty-five mosquitoes were triturated per ml of virus suspension with a chilled mortar and pestle. Mosquito-free virus controls were "triturated" in a similar fashion. Both control and mosquito suspensions were centrifuged for 1 hour at 10,000 x g at 4°C.

In the first experiment, Aedes aegypti mosquitoes were suspended in diluent containing approximately 10^4 plaque-forming units (PFU) of prototype dengue types 1-3 and chikungunya (Chik) viruses. After centrifugation, serial 10-fold dilutions were made and inoculated into mice and cell cultures. Infection of LLC-MK_2 cells by these viruses was inhibited (table 32). This inhibition was reversible upon dilution. As a result, plaque numbers in each ten-fold dilution tended to remain the same and, with the exception of dengue-1 virus, plaque numbers in the mosquito-virus mixtures and in the controls were about the same after 1000-fold dilution. The results of inoculation of mice with these control and mosquito-virus mixtures were only suggestive of inhibitory activity (table 33). A few more mice inoculated with mosquito-virus mixtures survived than did the controls. The mean survival time of the mosquito-virus mixture group was observed to be somewhat longer than the virus control group.

Table 32. Inhibition of dengue and chikungunya virus plaque production in LLC-MK_2 cell cultures by Aedes aegypti suspensions. Inhibition of test virus doses was reversible upon dilution.

Prototype Virus	Treatment	Dose	Dose-1	Dose-2	Dose-3
Dengue-1	Control	TN (6,400)1/	TN (640)	64	22
	Mosq.	17	4	1	0
Dengue-2	Control	TN (80,000)	TN (8,000)	TN (800)	80
	Mosq.	TN	89	76	53
Dengue-3	Control	TN (4,800)	TN (480)	48	14
	Mosq.	62	58	41	12
Chik	Control	TN (3,900)	(390)	39	2
	Mosq.	32	37	33	1

1/ TN=too numerous to count (plaque count calculated).

The second experiments were done in order to estimate how much virus loss might be anticipated under the usual conditions of virus recovery from mosquitoes. Ten-fold serial dilutions of local, low-passage dengue types 1-4, Japanese encephalitis (JE) and chik viruses were made first. Mosquitoes were then suspended in each dilution, and with controls, centrifuged and inoculated as before. Dengue 1-4 and chik virus infection of LLC-MK_2 cells was inhibited 10 to 2,300-fold by suspensions of A. aegypti mosquitoes (table 34). These viruses could not be simultaneously tested for in mice because of their very low mouse

Salivary gland fluorescence	aegypti	−	±	NT
	albopictus	±	+	±

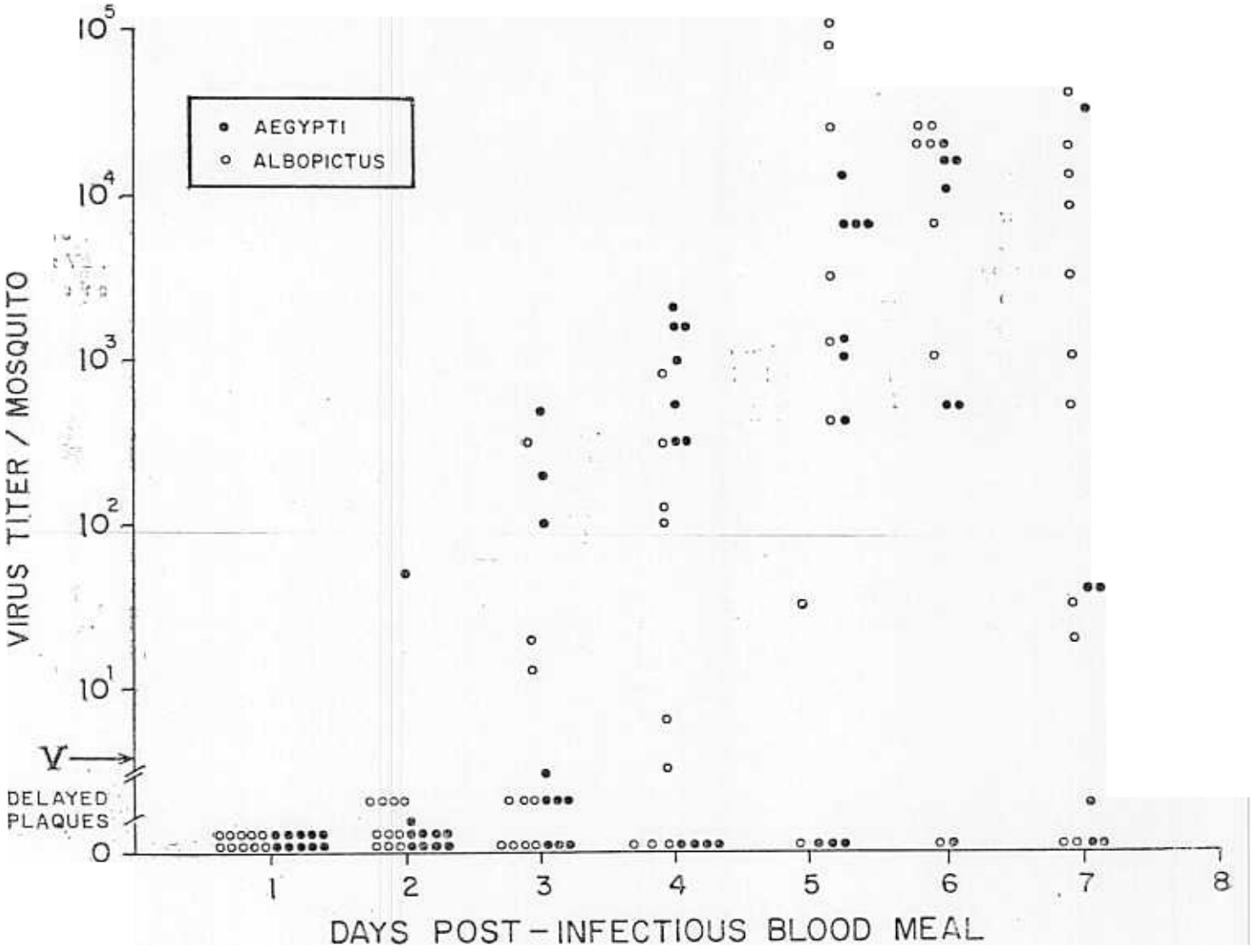

Figure 11.

Table 33. Mosquito–virus mixtures and mosquito–free controls inoculated intracerebrally and intraperitoneally into 1–day–old suckling mice. These are the same materials inoculated simultaneously into LLC–MK_2 cells (Table 32). Mouse mortality is indicated by number dying over the total number inoculated. Mean survival time and range of survival time are in days.

Prototype Virus	Virus Dose			Virus Dose–1			Virus Dose–2			Virus Dose–3		
	Mouse Mort.		Range Surv.	Mouse Mort.	Mean Surv.	Range Surv.	Mouse Mort.	Mean Surv.	Range Surv.	Mouse Mort.	Mean Surv.	Range Surv.
Dengue 1 Control	16/16		7–9	16/16	7.6	6–9	16/16	8.9	8–10	15/15	8.9	9–10
Dengue 1 + Mosq.	16/16	7.9	7–9	4/15	8.8	8–10	13/16	9.3	8–1	1/16	10	10
Dengue 2 Control	16/16	6.1	5–7	16/16	6.3	5–7	16/16	6.7	6–8	16/16	7.6	7–9
Dengue 2 + Mosq.	16/16	6.4	6–7	16/16	7.4	6–8	16/16	7.2	6–8	16/16	8.1	7–9
Dengue 3 Control	16/16	9.0	9	16/16	9.9	8–1	16/16	10.1	10–13	9/14	1.9	11–12
Dengue 3 + Mosq.	16/16	9.2	9–10	16/16	10.1	9–11	13/15	11.4	10–13	10/16	2.1	11–13
Chikungunya Control	16/16	3.0	3	15/15	3.0	3	15/15	3.1	3–4	12/16	3.7	3–4
Chikungunya + Mosq.	16/16	3.3	3–4	15/15	3.5	3–4	14/16	.8	3–4	8/15	.6	3–5

infectivity. Instead, low mouse passage strains of dengue viruses types 1—3 were tested with A. aegypti and no inhibition of infection was observed. In the case of the local strain of chik virus, no inhibition was detected either in mice or in cell cultures. JE virus infection of LLC—MK_2 cells was inhibited 12—to 64—fold by suspensions of C. tritaeniorhynchus and C. gelidus (table 35). No inhibition was observed when these suspensions were inoculated into suckling mice.

The influence of the numbers of individual mosquitoes in the pool on inhibitor concentration and subsequent reduction in infectivity has not yet been determined, but may be important. In this laboratory, where dengue viruses were recovered from pools of 25 female A. aegypti, only 9 of 29 strains were detected by direct plaque formation. The remainder were detected only after a period of maintenance as fluid cultures as done in the delayed plaque technique. Where pools of 1—10 individual A. aegypti and A. albopictus were tested, however, 16 strains of 25 were detected by direct plagues. While observed differences may have been due to virus strain variation, the difference in pool size and thus concentration of inhibitors is a possible explanation.

Table 34. Inhibition of plaque production by various concentrations of low—passage dengue virus by Aedes aegypti suspensions in LLC—MK_2 cells.

Dengue Type	Treatment	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Dengue—1	Control	TN (87,000)1/	TN (8,700)	TN (870)	78	
	Mosq.	35	19	0	0	
Dengue—2	Control		TN (15,700)	TN (1,570)	157	20
	Mosq.		91	5	1	0
Dengue—3	Control	TN (9,200)	TN (920)	92	30	
	Mosq.	TN	53	5	0	
Dengue—4	Control	TN (800)	80	19	1	
	Mosq.	87	8	1	0	

1/ TN=too numerous to count (plaque count calculated)

Table 35. Inhibition of plaque production by various concentrations of Japanese encephalitis virus in LLC—MK_2 cell cultures by suspensions of normal Culex tritaeniorhynchus and C. gelidus.

	Plaque Numbers at Dilutions			
Mosquito	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Control	TN (1,900) 1/	190	26	3
C. gelidus	79	16	0	0
C. tritaeniorhynchus	72	3	0	0

1/ Plaques too numerous to count (plaque count calculated).

Antigenic Variation in Strains of Japanese Encephalitis Virus from Various Geographic Areas

Antigenic differences among arbovirus strains from different geographic areas have been taken as evidence of ecologic isolation. A preliminary attempt was made to determine if antigenic variation was

Table 36. Homologous and heterologous 4 week post vaccinal serum titers following incubation of serum—JE virus mixtures at 3 different times.

Monkey no. (Vacc. virus strain)	V—140 (Taiwan)			V—137 (Thai)			V—142 (India)			V—139 (Sarawak)			V—144 (Japan)		
Incubation period (min at 37°C)	0*	45	90	0	45	90	0	45	90	0	45	90	0	45	90
Virus															
H—390 (Taiwan)	24	170	410	140	320	490	530	2600	5500	250	1800	2900	26	160	230
BKM 983/63 (Thai)	15	110	160	76	225	515	400	3200	4500	220	2300	3700	32	140	410
12753 (India)	32	350	640	48	350	550	400	2400	3500	225	1000	2300	75	200	450
MS 64/49 (Sarawak)	12	59	180	19	130	210	25	700	2100	370	3200	3800	32	120	270
Sasazaki (Japan)	40	100	240	40	300	640	450	2700	3000	300	1500	3400	22	320	1000

* Serum—virus mixtures inoculated immediately after mixing.

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Monkey no. (Vacc. virus strain)	V—140 (Taiwan)		V—137 (Thai)		V—142 (India)		V—139 (Sarawak)		V—144 (Japan)	
Incubation period (min at 37°C)	0*	90	0	90	0	90	0	90	0	90
Virus										
H—390 (Taiwan)	52	120	30	450	250	3300	70	640	13	100
BKM 983/63 (Thai)	32	270	35	540	270	6400	51	670	22	130
12753 (India)	20	170	36	280	240	2900	64	500	30	185
MS 64/49 (Sarawak)	30	170	20	175	320	2300	50	420	21	150
Sasazaki (Japan)	30	300	27	700	320	4800	94	600	10	320

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detectable among Japanese encephalitis (JE) virus strains from Japan, Taiwan, Thailand, India and Sarawak. One strain from each location in low (3—4) suckling mouse passage was used. Single—injection 4 and 11 week post-inoculation antisera was prepared in monkeys. Homologous and heterologous plaque reduction neutralization titers in LLC—MK_2 cell cultures were determined. Serum-virus mixtures were plaque assayed immediately after mixing (0 min) and after 45 and 90 min of incubation at 37°C. Adsorption time was reduced to 30 min to minimize the effects of neutralization during this period. Homologous and heterologous titers are shown in tables 36 (4 week sera) and 37 (11 week sera). The only differences detected were the failure of Thai and India strain antisera to neutralize Sarawak virus to the same titers as the homologous or other heterologous viruses (figs. 12 and 13). This slight difference was not reciprocal, nor was it apparent with the 11 week sera, and will have to be confirmed by additional tests. Thus, by the methods employed, JE strains from Continental Asia and nearby northern islands appear to be similar. Sarawak, if the observed slight antigenic differences are real, appears to be somewhat virologically isolated from the other areas. This is not particularly surprising in view of the evidence of ecological isolation of its vertebrate fauna.

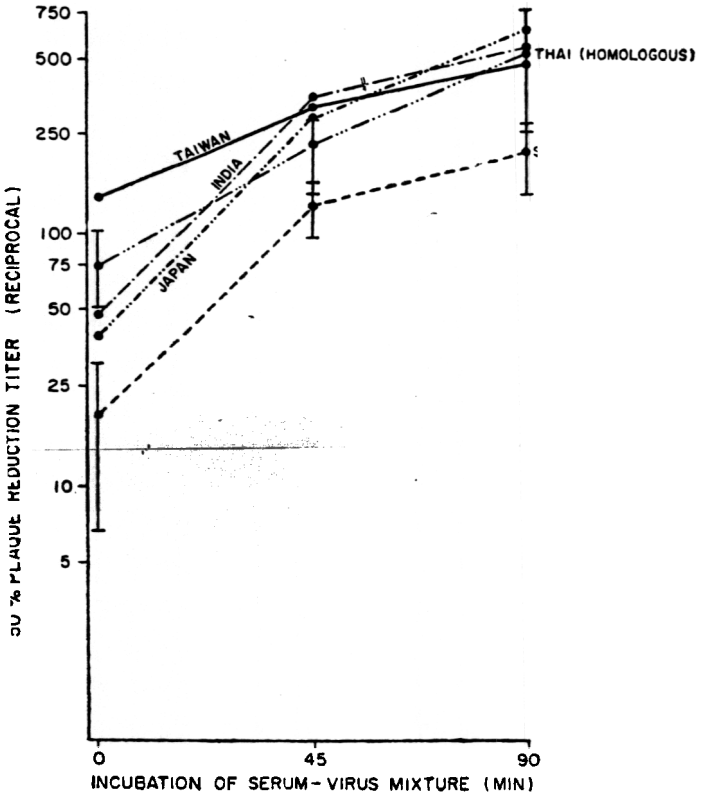

Figure 12.

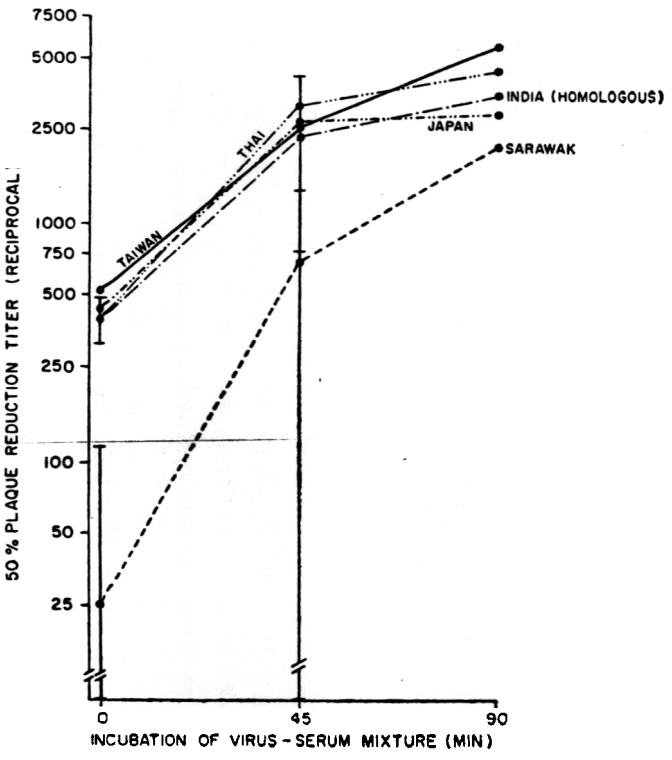

Figure 13.

Studies on the Ecology of Japanese Encephalitis Virus at Bang Phra, 1966–1968

Evidence for year round transmission of Japanese encephalitis (JE) virus at Bang Phra has been presented in earlier reports. Studies establishing relationships between wild vertebrates, horses, man, mosquitoes and the virus have continued.

The Red Cross Farm, operated by the Pasteur Institute to produce horse antivenin is 75 km SE of Bangkok at 50 m altitude, 13°12′ N by 100°57′ E in Sriracha District, Cholburi Province. It lies on a plain used for agriculture between wooded hills fronting a reservoir to the east and mangroves and villages along the gulf coast 1.5 km to the west. Most of the 16 hectares of the farm is in close–cropped pasture with scattered large rain trees and mangoes through which 130 horses run during the morning. The rest of the time the horses are kept in large stables. Three large fields comprising 4 hectares are devoted to tall grass which is sprinkled and hand cut to feed the horses. A fence boundary is covered with bushes and vines. Another long boundary is formed by a small river, carrying effluent from a tapioca mill. It is lined with thickets of tall bamboo and the banks are covered with dense secondary woods. There are a spring and several small creeks running through thickets, tall grass and banana groves. Workers' housing, laboratory buildings, concrete drinking troughs, pens, residences, a high power line and shady mango groves complete the picture of a small area intensively used by man and beast. It provides diversity of habitats to support a remarkably varied fauna both of mosquitoes and of small vertebrates.

Approximately 150 persons live at the horse farm, and there are 39 dogs, 205 chickens, 20 ducks, and 14 cats. Doves and rabbits are kept in cages. The village of Bang Phra a kilometer to the west has 334 houses and about 2000 persons; there are the usual domestic and commensal animals except for pigs. We know of only one small piggery there.

Surrounding the serum farm are plowed tapioca fields some of which occasionally revert to dense weeds and grass, coconut groves, fruit orchards, and a dairy of 450 head. The closest rice paddies are 2 and 4 km away at the edges of the reservoir. Adjacent natural habitats are mangrove swamps on the gulf coast. Portions of the coast have a sandy beach behind which are some rain pools. Khao Chalak, rising 313 m just south of the horse farm, has considerable mixed deciduous forest remnants, with a bamboo understory. Original evergreen and mixed deciduous forest is found 10 km northeast of the horse farm on a small mountain range including Huay Kum waterfall and Khao Khieo of 798 m altitude. It has a rich fauna even though large animals have been hunted out.

The first year of the study (1966) experienced a greater than normal rainfall of 1567 mm, whereas the second was unusually dry at 814 mm. From 1961 through 1967, the average annual rainfall was 1206 mm arriving mostly from May through October.

In the Bang Phra area in general, and on the horse farm specifically, wild animals were captured in 13 general habitat types (figures 14 and 15). The various animals, caught by hand, noosed netted, and live-trapped were bled from the heart except that birds were bled from the right jugular vein and mice from the retro–orbital sinus after injecting 0.3 ml of saline into the body cavity of the mice. A few of the smallest birds died but most survived bleeding. Blood samples ranged from 0.1 ml to 1 ml according to the size of the animal and small samples were diluted 1:4–1:10. (Diluent: slightly alkaline with bicarbonate, BME tissue culture medium with 20% heat inactivated fetal bovine serum plus 500 units of penicillin and 250 micrograms of streptomycin per ml of final volume). Blood was drawn into a dry syringe with a dry needle and let into a centrifuge tube, diluent was added, diluted blood was later rimmed down and moderately spun to pack the cells. Serum was drawn off, put into a sterile vial labelled with type written adhesive tape, sealed with plastic paraffin, and placed on dry ice. Meanwhile the donor animal was identified, toe–clipped or supplied with an aluminum numbered band or ear tag, weighed, measured, sex determined, inspected to determine the reproductive condition and these data, along with date and specific location of capture were recorded on a punch card before the animal was released.

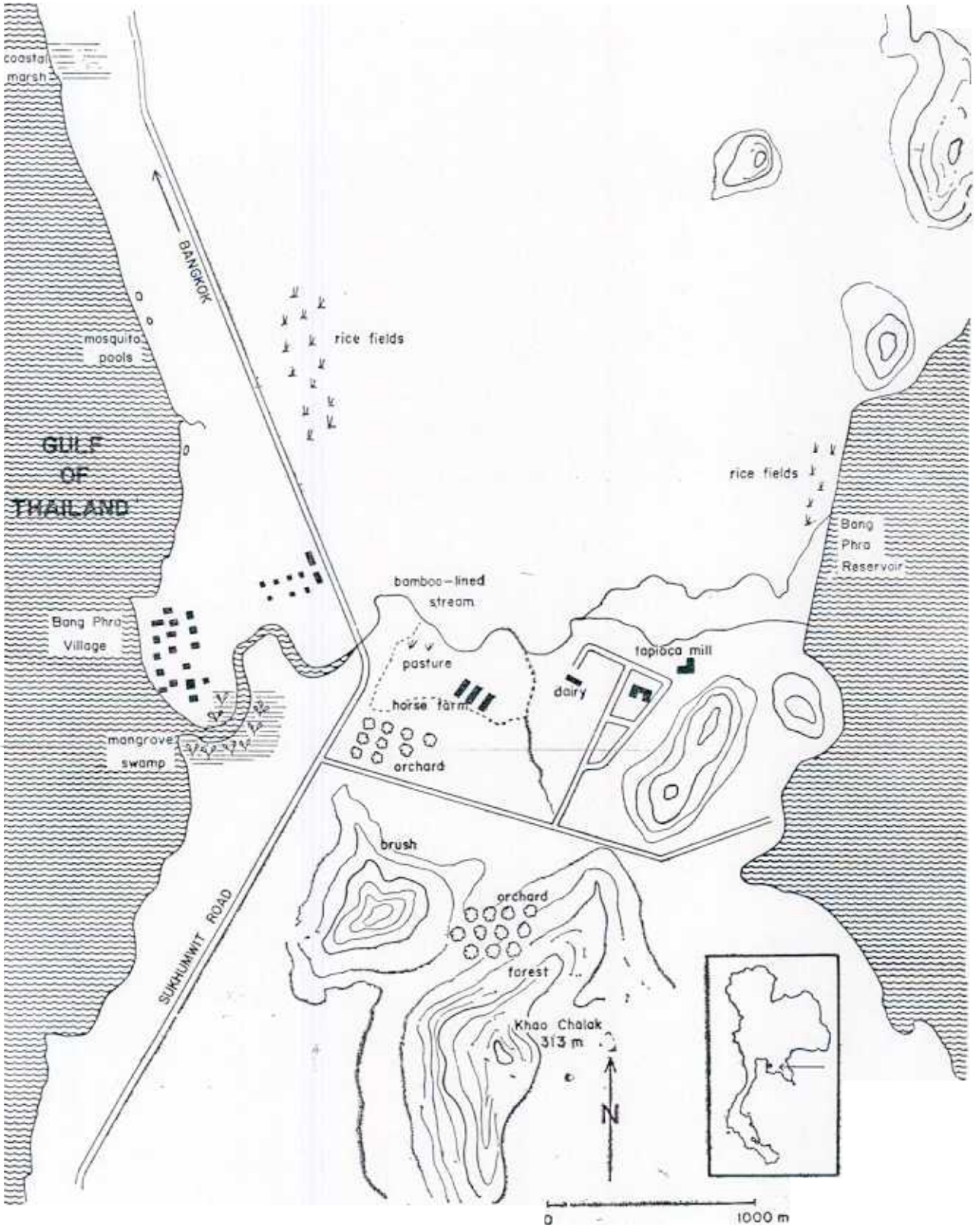
coastal marsh
BANGKOK
mosquito pools
rice fields
GULF OF THAILAND
rice fields
Bang Phra Reservoir
bamboo-lined stream
Bang Phra Village
pasture
tapioca mill
horse farm
dairy
mangrove swamp
orchard
brush
orchard
SUKHUMVIT ROAD
forest
Khao Chalak 313 m
N
0
1000 m

Figure 14.

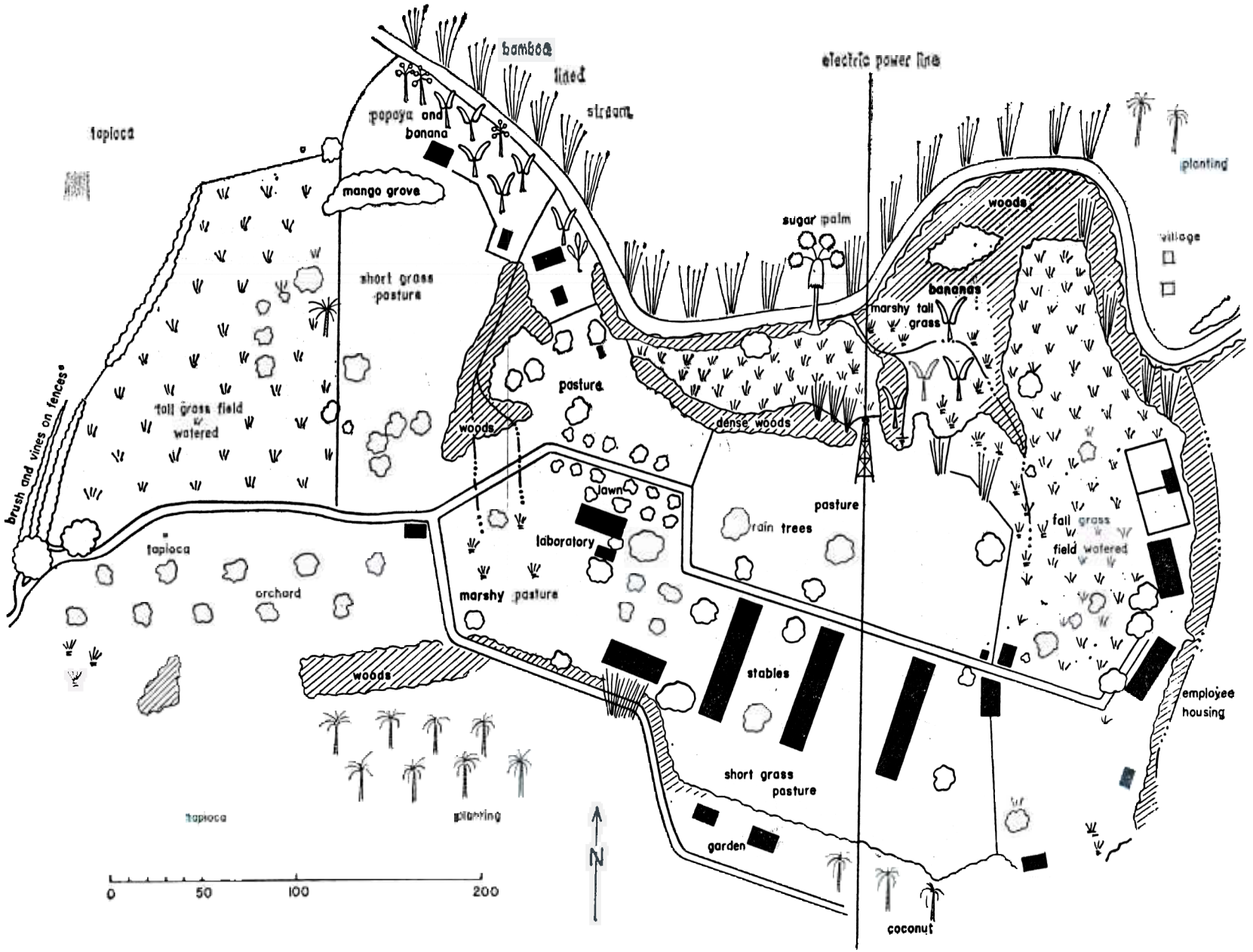
bamboo lined stream
electric power line
tapioca
papaya and banana
mango grove
short grass pasture
sugar palm
woods
planting
village
bananas
marshy tall grass
brush and vines on fences
tall grass field watered
pasture
woods
dense woods
lawn
pasture
rain trees
tapioca
laboratory
tall grass field watered
orchard
marshy pasture
woods
stables
employee housing
short grass pasture
garden
coconut
tapioca
planting
N
0
50
100
200

Figure 54

In the laboratory, sera were tested for neutralizing antibody in the BHK–21 cell metabolic inhibition (MI) test. Sera neutralizing 30–100 TCD_{50} of virus (2–3 wells of 3 wells protected) were considered to contain specific antibody. To date, 2,802 individual blood specimens representing 114 species captured in and around the horse farm have been tested for JE virus antibody. These species are characteristically found in 13 habitat types (table 38). As in most tropical communities, species tended to be numerous and individual of any given species were comparatively few. Man's disturbance of the area has, however, altered the ecology of the area to permit 18 of these species to become relatively abundant.

Evaluation of the serological data in terms of the species infected and the probable time and place of their infection presents a picture of ubiquitous low level, continuous infection. There is no evidence for nodality on the study area; infected species were found in every habitat type (table 39). Overall, 68 of the 114 species had JE virus antibody but the plethora of species, paucity of individuals and irregularity of sampling made comparison of prevalence by habitat type unrealistic.

No evidence of epizootic virus transmission among wild vertebrates was found. Overall, antibody was detected in 649 of 2802 individual wild vertebrates tested. Monthly antibody prevalence rates were low but appeared to fluctuate (0–38%). Although changes in species composition of monthly sampling and the comparatively low numbers tested during some months limits the reliability of these monthly prevalence estimates, the data are suggestive of enzootic rather than explosive epizootic transmission. There are probably adequate numbers of susceptible individuals and vectors present in the area to support enzootic transmission. All but one of the 18 abundant species are represented by serologically positive individuals (table 39). These species are all relatively short lived, have high rates of population turnover and most (15 species) are year round residents of the area. The breeding season on the study area is more protracted than that of temperate regions. Breeding in birds, for example, is more variable and less synchronous among individuals. Display and singing begins in January, nesting (judged from the incubation patch in adults and time of appearance of juveniles in the population) extends from February through July. This extended breeding probably serves to continuously add susceptible individuals to the population over a fairly long period of time (6 months), a situation more likely to sustain enzootic than epizootic transmission.

There is, however, some evidence for epizootic "spillover" into the farm horse population. Groups of horses from Australia were brought into the farm in May of 1966 and June of 1967. The first group of horses developed JE virus antibody very rapidly; when sampled at the end of a month all had become infected (figure 16). The second group of horses did not develop antibody as quickly. From July–October only 22 of the horses became serologically positive. Within the following two months and additional 70% were infected (figure 16). These periods of high incidence of horse infection suggest that virus transmission is more frequent during certain times. Unlike epizootic periods in temperate areas, no corresponding increase in infection of the two mosquito vectors, C. tritaeniorhynchus and C. gelidus, which are doubtless responsible for transmission of JE virus to horses, has been observed.

There is apparently less infection of man than there is of horses. A serological survey was conducted among 995 school children resident in the Bang Phra area. Age specific neutralizing antibody prevalence was about the same for all age groups (figure 17). This is in striking contrast to the age specific neutralizing antibody to chikungunya virus, in the same children, where prevalence increased gradually with age from 35% in the 6 year old group to 70% in the 15 year olds (figure 18). JE virus antibody was lower that than to chikungunya virus, varying from 7% in the 8 year old group to 21% in the 12 year old group. Either JE virus infection of these children has been so rare that only one or two occurrences of "spillover" has produced this flat curve, or yearly infection was of such low magnitude that the sample sizes of each group were too small to detect the small increases in the percentage of positives.

Serological surveys may indicate which species are being naturally exposed to the virus but provide no insight into the ability of any given species to support virus replication and serve as a source of infectious blood meals for arthropod vectors. Experimental infection and determination of duration and amplitude of viremia provides useful clues as to the reservoir potential of species suspected on serological grounds. Accordingly, a number of abundant resident wild vertebrate species having relatively

Table 38. Prevalence of neutralizing antibody to Japanese encephalitis (JE) and Wesselsbron (WESS) viruses in Bang Phra vertebrates, 1966–1967.

Species	Abundance[1]	Seasonal Status[2]	Neut. Antibody JE Pos.[3]/Total (%)	Neut. Antibody WESS Pos./Total (%)
Fresh Water				
Phrynoglossus martensii	c	R	0/1 (0)	0/1 (0)
Rana tigerina	c	R	2/28 (7)	0/28 (0)
Rana limnocharis	a	R	1/10 (10)	1/11 (9)
Rana erythraea	c	R	0/13 (0)	0/13 (0)
Rana macrodactyla	u	R	0/2 (0)	0/2 (0)
Natrix flavipunctata	u	R	0/1 (0)	0/1 (0)
Enhydris enhydris	a	R	0/6 (0)	0/6 (0)
Enhydris plumbea	u	R	0/2 (0)	0/2 (0)
Coastal Marsh				
Rostratula benghalensis	u	R	0/1 (0)	0/1 (0)
Charadrius dominicus	c	W	0/1 (0)	0/1 (0)
Charadrius dubius	c	M	0/1 (0)	—
Charadrius alexandrinus	c	M	0/3 (0)	1/1 (100)
Actitis hypoleucos	c	W	2/3 (67)	1/3 (33)
Calidris subminuta	a	M	6/14 (43)	0/9 (0)
Bamboo–lined Stream				
Ardeola ralloides bacchus	f	W	0/1 (0)	0/1 (0)
Ixobryschus cinnamomeus	f	R	0/2 (0)	0/2 (0)
Amaurornis phoenicurus	u	R	1/1 (100)	0/1 (0)
Alcedo atthis		R	0/1 (0)	0/1 (0)
Halcyon smyrnensis		R	1/9 (11)	0/7 (0)
Halcyon pileata		W	2/5 (40)	0/4 (0)
Motacilla alba		W	1/1 (100)	0/1 (0)
Motacilla caspica		W	2/3 (67)	0/3 (0)
Motacilla flava		W	4/6 (67)	1/6 (17)
Rattus berdmorei		R	5/8 (63)	0/9 (0)
Marshy Tall Grass				
Phragamaticola aedon	c	W	4/16 (25)	3/12 (25)
Acrocephalus arundinaceus	c	W	4/9 (44)	0/5 (0)
Acrocephalus agricola	r	W	0/1 (0)	0/1 (0)
Bandicota indica	u	R	18/38 (47)	7/23 (30)
Tall Grass				
Xenopeltis unicolor	u	R	0/2 (0)	0/2 (0)
Turnix tanki	r	R	0/1 (0)	0/1 (0)
Turnix suscitator	f	R	0/3 (0)	0/4 (0)
Lonchura punctulata	f	R	10/53 (19)	1/51 (20)
Mus cervicolor	a	R	24/69 (34)	2/54 (4)
Mus famulus	c	R	7/20 (35)	1/20 (5)
Short Grass				
Bufo melanostictus	a	R	1/48 (2)	4/48 (8)
Capella gallinago	u	M	0/1 (0)	0/1 (0)

Table 38. (Continued)

Species	Abundance[1]	Seasonal Status[2]	Neut. Antibody JE Pos.[3]/Total (%)	Neut. Antibody WESS Pos./Total (%)
Short Grass				
Capella stenura	c	M	2/3 (67)	0/3 (0)
Streptopelia chinensis	f	R	0/2 (0)	1/2 (50)
Streptopelia tranquebarica	c	R	0/2 (0)	0/2 (0)
Mirafra assamica	c	R	3/11 (27)	2/10 (20)
Saxicola torquata	r	W	1/1 (10)	0/1 (0)
Anthus novaeseelandiae	c	R/W	1/5 (20)	1/4 (25)
Ground near Shade				
Kaloula pulchra	u	R	0/1 (0)	0/1 (0)
Glyphoglossus molossus	r	R	0/1 (0)	0/1 (0)
Mabuya multifasiata	u	R	0/1 (0)	0/1 (0)
Oligodon quandrilineatus	u	R	0/1 (0)	0/1 (0)
Geopelia striata	u	R	2/4 (50)	0/4 (0)
Copsychus saularis	a	R	31/100 (31)	6/93 (6)
Copsychus malabaricus	c	R	0/3 (0)	0/3 (0)
Dendronanthus indicus	f	W	0/3 (0)	0/4 (0)
Anthus hodgsoni	u	W	1/2 (50)	0/2 (0)
Rattus rajah	c	R	1/4 (25)	0/3 (0)
Herpestes javanicus	u	R	0/3 (0)	0/3 (0)
Banana Grove				
Rousettus leschenaulti	c	R	3/19 (16)	2/17 (12)
Pteropus lylei	c	R	0/2 (0)	0/2 (0)
Cynopterus brachyotis	a	R	32/294 (10)	4/231 (1)
Eonycteris spelaea	c	R	3/8 (38)	0/11 (0)
Brush Thickets				
Calotes versicolor	c	R	8/14 (57)	0/14 (0)
Calotes mystaccus	f	R	8/13 (61)	1/13 (8)
Centropus toulou	u	W/R	1/1	
Timalia pileata	f	R	0/11 (0)	0/1 (0)
Erithacus calliope	r	W	0/1 (0)	0/1 (0)
Erithacus svecicus	r	W	—	
Erithacus cyane	u	W	0/3 (0)	0/3 (0)
Orthotomus sutorius	c	R	3/11 (27)	0/9 (0)
Prinia rufescens	t	V	0/1 (0)	0/1 (0)
Tupaia glis	u	R	1/4 (25)	0/3 (0)
Menetes berdmorei	c	R	8/24 (33)	1/18 (5)
Rattus rattus	a	R	183/462 (40)	70/395 (18)
Woods and Shady Trees				
Dryophis nasutus	c	R	0/2 (0)	0/2 (0)
Accipiter badius	u	R	1/4 (25)	0/3 (0)
Cacomantis sonneratii	u	V	0/2 (0)	0/2 (0)
Cacomantis merulinus	f	W	2/7 (28)	0/7 (0)
Phoenicophaeus fristis	r	V	—	1/1 (100)
Otus scops	u	W	0/1 (0)	0/1 (0)
Otus bakkamoena	f	R	0/2 (0)	0/2 (0)
Aegithina tiphia	f	R	1/2 (50)	1/2 (50)

Species	Abundance[1]	Seasonal Status[2]	Neut. Antibody JE Pos.[3] / Total (%)	Neut. Antibody WESS Pos. / Total (%)
Woods and Shady Trees				
Pycnonotus atriceps	r	V	1/4 (25)	0/3 (0)
Pycnonotus aurigaster	c	R	5/28 (18)	0/29 (0)
Pycnonotus goiavier	a	R	68/314 (22)	16/231 (7)
Pycnonotus blanfordi	a	R	46/314 (15)	16/255 (6)
Crypsirina temia	f	R	2/10 (20)	2/7 (28)
Macronous gularis	c	R	1/9 (11)	0/8 (0)
Phylloscopus fuscatus	u	W	2/3 (67)	0/3 (0)
Phylloscopus schwarzi	r	W	0/1 (0)	0/1 (0)
Phylloscopus borealis	f	W	0/2 (0)	0/1 (0)
Phylloscopus coronatus	f	M	0/1 (0)	0/1 (0)
Phylloscoyus tenellipes	u	W	0/3 (0)	0/3 (0)
Muscicapa zanthopygia	u	M	1/3 (33)	0/2 (0)
Muscicapa parva	f	W	1/3 (33)	0/3 (0)
Rhipidura javanica	c	R	7/66 (11)	2/61 (3)
Hypothymis azurea	u	R	—	0/1 (0)
Dicaeum cruentatum	c	R	0/2 (0)	0/2 (0)
Trees in Open				
Athene brama	f	R	0/1 (0)	
Caprimulgus macrurus	c	R	0/1 (0)	
Upupa epops saturata	r	W	1/3 (33)	0/3 (0)
Upupa epops longirostris	r	R		
Lanius cristatus	c	W	17/33 (51)	3/30 (10)
Lanius nasutus	f	R	7/15 (47)	4/15 (27)
Sturnus contra	c	R	9/47 (19)	6/37 (16)
Sturnus nigricollis	a	R	2/9 (22)	0/8 (0)
Sturnus burmanicus	r	R	1/2 (50)	0/1 (0)
Sturnus javanicus	a	R	1/33 (3)	9/30 (30)
Sturnus tristis	a	R	3/25 (12)	3/17 (18)
Nectarinia jugularis	f	R	0/2 (0)	—
Ploceus philippinus	c	S	4/88 (2)	2/76 (4)
Passer flaveolus	a	R	48/161 (30)	9/140 (6)
Buildings				
Hemidactylus frenatus	a	R	7/14 (50)	0/14 (0)
Gekko gecko	c	R	1/6 (17)	0/6 (0)
Passer montanus	a	R	5/49 (10)	1/31 (3)
Rattus norvegicus	u	R	3/9 (33)	13/21 (62)
Rattus exulans	c	R	10/22 (45)	19/59 (32)
Aerial				
Cypsiurus parvus	c	R	0/4 (0)	0/5 (0)
Merops leschenaulti	c	R	0/4 (0)	0/4 (0)
Merops orientalis	c	R	0/23 (0)	0/23 (0)
Merops viridis	r	R	0/5 (0)	0/4 (0)
Hirundo rustica	a	W	2/11 (18)	1/11 (9)
Dicrurus adsimilis	a	W	2/7 (29)	0/5 (0)
Dicrurus leucophaeus	r	W	1/2 (50)	0/2 (0)
Dicrurus paradiseus	f	R	0/1 (0)	1/2 (50)
Artamus fuscus	c	R	1/2 (50)	0/1 (0)

[1] a=abundant, c=common, f=frequent, u=uncommon, r=rare.

[2] R=year round resident, M=passage migrant, W=winter resident, S=summer resident, V=vagrant.

[3] Sera neutralizing 50% of 30—100 metabolic inhibition test LD_{50} of virus.

Table 39. Prevalence of antibody to Japanese encephalitis virus and to Wesselsbron virus in species found in various habits, by relative abundance.

Habitats	No. Species Positive by Relative Abundance											
	Japanese Enceph. Virus						Wesselsbron Virus					
	A[1]	C[2]	F[3]	U[4]	R[5]	Total	A	C	F	U	R	Total
Fresh Water	1/2[6]	1/3		0/3		2/8	1/2	0/3		0/3		1/8
Coastal Marsh	1/1	1/4		0/1		2/6	0/1	2/3		0/1		2/6
Bamboo–Lined Stream		4/4	1/4	1/1	1/1	7/10		1/4	1/4		0/1	1/9
Marshy Tall Grass		2/2		1/1	0/1	3/4		1/2		1/1	0/1	2/4
Tall Grass	1/1	1/1	1/2	0/1	0/1	3/6	1/1	1/1	1/2	0/1	0/1	3/6
Short Grass	1/1	3/4	0/1	0/1	1/1	4/8	1/1	2/4	1/1	0/1	0/1	4/8
Ground Near Shade	1/1	1/2	0/1	2/6		4/10	1/1	0/2	0/1	0/6		1/10
Banana Grove	1/1	2/3				3/4	1/1	1/3				2/4
Brush Thickets	1/1	3/3	1/2	2/3	0/1	7/10	1/1	1/3	1/2	0/2	0/1	3/9
Woods and Shady Trees	2/2	3/5	4/7	3/6	1/2	13/22	2/2	2/5	1/3	0/7	1/3	5/20
Trees in Open	4/4	3/4	1/3		1/1	9/12	3/4	3/3	1/1		0/1	7/9
Buildings	2/2	2/2		1/1	5/5	1/2	1/2		1/1			3/5
Aerial	2/2	1/4	0/1		1/2	4/9	1/2	0/4	1/1		0/2	2/9
Total	17/18	28/41	8/21	10/24	5/10	68/114	13/18	15/40	6/15	2/23	1/11	37/107

1/ Abundant species.
2/ Common species.
3/ Frequent species.
4/ Uncommon species.
5/ Rare species.
6/ Species neutralizing 30–100 LD_{50} virus/total species tested.

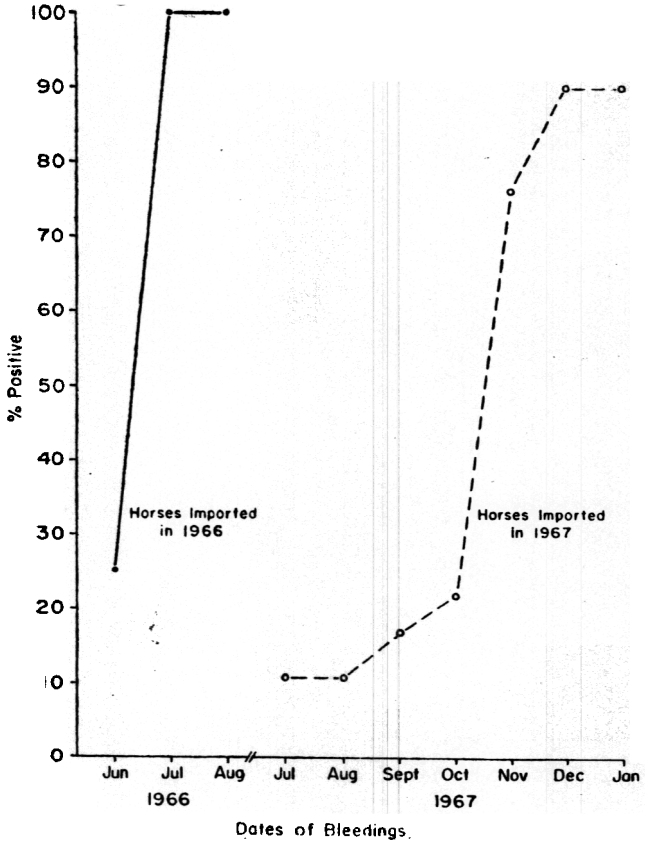

Figure 16.

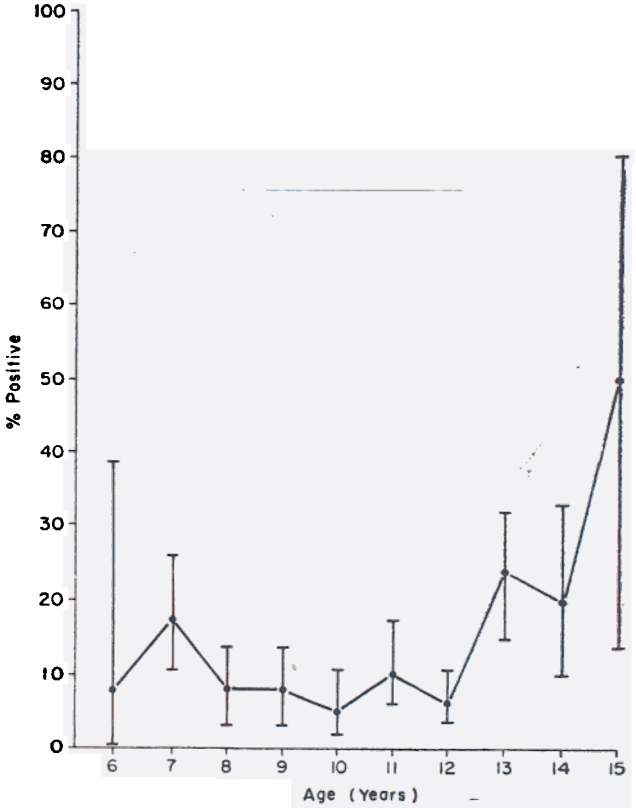

Figure 17.

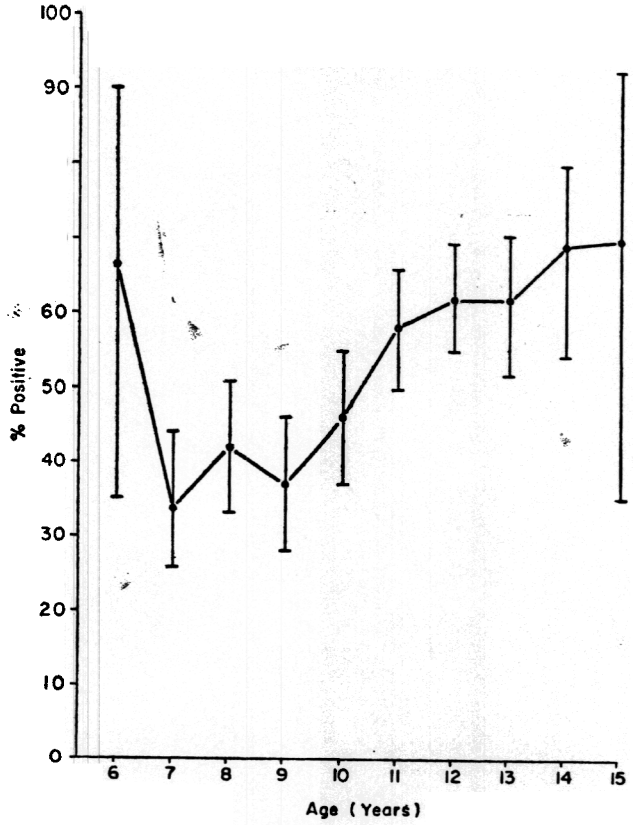

Figure 18.

high antibody prevalence rates were tested in the laboratory. Those without antibody to JE virus were inoculated with 300—3000 LLC—MK_2 cell plaque forming units of Bang Phra strain JE virus in the 3—4 suckling mouse passage. Blood specimens drawn on 10 consecutive days were tested for virus in suckling mice and in either BHK—21 cell tube cultures or in LLC—MK_2 cells by mean of direct and delayed plaques. All viruses recovered from the blood specimens were confirmed as JE virus by neutralization with reference antiserum. Birds were bled from the external jugular vein, mice from the retroorbital sinus, bats and rats from the heart and Calotes lizards by means of Aedes aegypti mosquitoes. The results are shown in table 40. Mammals (mice, rats and bats) did not develop detectable viremias, but did respond, except for 5 of 6 juvenile Mus famulus, with neutralizing antibody. The results of attempts to infect species of birds having relatively high antibody prevalence rates in the field were not consistant. Two species of Bulbuls (Pycnonotus blanfordi and P. goiavier) developed no detectable viremia and none of the Blanford's bulbuls developed neutralizing antibody. Two sparrow species abundant in the field developed persistant viremias of 5—7 days. Two of 2 pegu sparrows (Passer flaveolus), and 1 of 5 house sparrows (Passer montanus) circulated virus in their blood to 10^5 infectious units per ml. The lizards (Calotes versicolor) developed no detectable viremia. Because of their small size we were unable to bleed them serially from the heart. Daily blood specimens were drawn by permitting Aedes aegypti mosquitoes to feed upon them. The abdomens of the freshly engorged mosquitoes were triturated in a chilled mortar and pestle, suspended in high-serum (20%) tissue culture medium 199 and inoculated into mice and cell cultures. The failure to detect virus in the blood may have been due to insusceptibility of the species, to the very small volumes of blood tested each day (approximately 0.003—0.03 ml) or to the inhibitory action of mosquito suspensions on infection of the suckling mice or cell cultures.

Table 40. Viremia following subcutaneous inoculation of Japanese encephalitis into selected birds, mammals and reptiles.

Species	Common Name	No. Tested	Age	Virus Dose	Viremia	
					No. Viremic	Days Duration
Birds						
Passer flaveolus	Pegu sparrow	2	Ad	$10^{2.5}$	2	5,7
Passer montanus	House sparrow	5	Ad	$10^{2.5}$	1	5
Pycnonotus goiavier	Bulbul	10	Ad	$10^{2.5}$, $10^{3.5}$	0	
P. blanfordi	Bulbul	4	Ad	$10^{2.5}$	0	
Mammals						
Mus cervicolor	Mouse	2	Ad	$10^{2.5}$	0	
Mus famulus	Mouse	2	Ad	$10^{2.5}$	0	
		5	Juv	$10^{2.5}$	0	
Rattus rattus	Roof rat	5	Ad	$10^{2.5}$	0	
Cynopterus brachyotis	Dog–faced Fruit bat	6	Ad	$10^{2.5}$	0	
Reptiles						
Calotes versicolor	Lizard	5	Ad	10^{3}	0	

It is difficult to explain why individuals of species that are apparently infected in the field failed to respond even with antibody when inoculated in the laboratory. This could have been due to insusceptibility of these individuals to mouse passaged virus, to administration of virus by artificial routes, or to individual variation in susceptibility. It was not known if individuals refractory to infection by inoculation in the laboratory are susceptible to infection following the bite of infected mosquitoes or if individuals which failed to develop detectable viremia but did respond with neutralizing antibody could have served as sources of infectious blood meals for vectors which are exquisitely susceptible to infection with the virus. Individuals which responded to JE inoculation with neutralizing antibody but not viremia may have already been immune, low titered preimmunization sera reacting as negative in the relatively insensitive MI test.

The validity of the serological results rests on the reliability of the MI test as a mean of determining past infection. Comparison of the MI, plaque reduction neutralization and hemagglutination–inhibitions during the development of the MI test have shown it to be highly specific, although of limited sensitivity. The high degree of specificity and freedom from nonspecific reactions have been suggested by the results of routine tests. Of nearly 3000 wild vertebrate sera tested to date, only one has neutralized all four viruses. Cross reactions within antigenic groups do not seem to be a serious problem. Only 72 of the 649 wild vertebrate sera neutralizing JE virus also neutralized Wesselsbron virus. During the months when sufficient numbers of resident species were tested to permit valid comparison, monthly changes in antibody prevalence rates to JE and Wesselsbron virus are not parallel, demonstrating that the MI test is capable of measuring the independent infection of the wild vertebrate population by two Group B viruses. Reactions of human sera appeared to be equally specific. Of the 128 sera from school children neutralizing JE virus and 117 neutralizing Wesselsbron virus only 24 neutralized both. Lack of marked increasing age specific antibody prevalence with these group B viruses also suggests that cross reactions with antibody to dengue viruses was not a problem.

Bang Phra Mosquito Study

Between July 1967 and March 1968 collections of larval and adult mosquitoes were made on and in the vicinity of the horse farm operated at Bang Phra (figures 14 and 15) by the Red Cross Society of Thailand. Collections of adult mosquitoes were made at weekly or more frequent intervals with 1) a New Jersey type light trap modified to capture live specimens, 2) while biting horses and 3) in bait traps containing a variety of species of small vertebrates and/or dry ice. The most abundant species captured in the light trap, which was located inside one of the stables are listed in table 41. Peaks in abundance of most of the species, as measured by light trap collections, occurred during the months of October and November. Secondary peaks in the numbers of Culex gelidus and Culex tritaeniorhynchus occurred in July and August, and a third peak in the numbers of C. gelidus collected occurred in January. During the previous season two peaks in numbers of these two species were observed during May—June and again in September—October. During the 1967—1968 season the total number of C. gelidus collected by light trap was again greater than that of C. tritaeniorhynchus. Table 42 summarizes the results of collections of mosquitoes biting horses between 1830 and 2100 hours at Bang Phra. By both the above types of collection it appears that the C. gelidus population in the Bang Phra area persists in greater density for a longer period of the year than does the C. tritaeniorhynchus population. Table 43 summarizes the results of collections of mosquitoes from lard—can type bait traps in which two species of birds (Passer flaveolus and Pycnonotus goiavier) and a lizard (Calotes versicolor) were used as bait animals. In addition, CO_2, in the form of dry ice, enclosed in perforated styrofoam boxes, was placed in some of the bait traps together with the bait animals; some traps contained CO_2 only. The presence of the CO_2 had a general effect in increasing the number of mosquitoes caught in all of the bait traps. No mosquitoes were collected from traps baited with Calotes versicolor alone, but significantly greater numbers of mosquitoes were collected from traps containing lizards and dry ice than from traps containing dry ice only. The bait—trap collections yielded significantly greater numbers of C. quinquefasciatus than did the collections from the light trap; this species was not attracted at all to the horses. On the other hand, Aedes lineatopennis, A. mediolineatus A. vexans and A. vigilax which came in large numbers to the light trap and to the horses, were not attracted to the bait traps, while C. gelidus and C. tritaeniorhynchus were present in small numbers in bait-trap collections in proportion to the other species represented in those collections (table 43). Culex fuscocephalus was present only in collections from the light trap.

A total of 64,130 mosquitoes of 23 species collected by the above methods were tested for the presence of virus, but all isolation attempts were negative during the 1967—1968 season.

The larval habitats of some of of the more abundant species of mosquitoes collected at Bang Phra during this report period are given in table 44. Two of the species listed, Aedes vigilax and Culex sitiens, were collected only in brackish pools on the sea coast-a distance of at least km. from the horse farm.

Isolation of Wesselsbron Virus in Thailand

Four strains of a group B arbovirus (BKM-367, BKM-589, BKM-448 and BKM-660) were recovered from Aedes mediolineatus and Aedes lineatopennis mosquitoes collected at the Bang Phra study site in June and July 1966. The details of the isolation of these agents and the preliminary serologic studies which indicated that they are group B arboviruses were reported in the Annual Progress Report prepared 15 April 1967. Additional studies by plaque reduction neutralization tests were carried out and the results of these tests, given in table 45, indicate that BKM—367, BKM—589, BKM—448 and BKM—660 are strains of the same virus. Neutralization test data previously reported indicated that these agents are not antigenically closely related to the group B arboviruses known to be present in Thailand i.e. the dengue viruses, Tembusu, and Japanese encephalitis viruses.

Toble 41. Female culicine mosquitoes collected per night (1800—0600) by light trap at Bang Phra, 1967—68.

Mosquito Species	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
Aedes lineatopennis	4	6	0	20	8	1	1	1	6
Aedes mediolineatus	27	32	58	239	76	1	1	1	9
Aedes vexans	7	11	12	483	132	2	23	20	270
Aedes vigilax	0	0	4	41	3	4	36	13	51
Culex annulus	0	0	96	0	115	258	196	43	60
Culex fuscocephalus	139	294	195	683	336	218	730	61	116
Culex gelidus	830	1166	458	1392	3300	1090	1774	422	530
Culex pseudovishnui	2	13	25	0	4	19	38	56	65
Culex quinquefasciatus	1	2	5	0	0	2	4	8	5
Culex sitiens	2	4	14	0	12	49	320	49	84
Culex tritaeniorhynchus	1875	760	407	1503	1356	316	154	141	293
Mansonia annulifera	8	7	0	45	24	3	34	25	26
Mansonia uniformis	25	10	0	61	38	108	550	131	177

Table 42. Culicine mosquitoes collected per hour biting horses at Bang Phra, 1967–68.

Mosquito Species	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
Aedes lineatopennis	0	1	<1	5	1.5	0	<1	1	2
Aedes mediolineatus	<1	4	3	7	2	0	0	<1	<1
Aedes vexans	<1	<1	<1	35	3	2	2	18	29
Aedes vigilax	0	0	0	5	<1	<1	3.5	19	6
Culex annulus	0	<1	1	2	1	0	1	<1	2
Culex gelidus	1	9	5	23	32	34	25	6	18
Culex pseudovishnui	0	1	1	0	<1	1	<1	1	<1
Culev sitiens	<1	<1	1.5	1	1.5	13	8	11	10
Culex tritaeniorhynchus	4	4	6	31	3	2	2	4	2.5
Mansonia uniformis	<1	<1	0	2	22	41	27	22	29

Table 43. Female culicine mosquitoes collected per trap–night (1800–0600) by bait traps at Bang Phra, November 1967–March 1968.

Mosquito Species	Passer	P. flaveolus + CO_2	Pycnonotus goiavier	P. goiavier + CO_2	Calotes versicolor	C. versicolor + CO_2	CO_2
Culex annulus	0	3	0	2	0	<1	<1
C. fuscanus	1	1	0	0	0	0	0
C. gelidus	<1	1	0	1	0	<1	<1
C. pseudovishnui	<1	14	0	14	0	4	3
C. quinquefasciatus	5	107	2	54	0	41	11
C. sitiens	3	97	1	33	0	20	11
C. tritaeniorhynchus	0	2	0	1	0	0	<1
Mansonia crassipes	<1	4	0	2	0	1	0

Table 44. Sources of culicine larvae collected at Bang Phra, August–September 1967.

Species	Habitat*						
	Ground pool	Wheel rut	Hoof print	Rice field	Stream margin	Ditch	Seepage bog
Aedes (Aedimorphus) mediolineatus	4	3	1				
vexans	2	1	1	1			
Aedes (Neomelanoconion) lineatopennis	1	1					
Aedes (Ochlerotatus) vigilax	6						
Culex (Culex) annulus	5		4		1	3	
Culex (Culex) fuscocephalus	27	14	16		2	2	2
Culex (Culex) gelidus	15	10	9		3	1	
Culex (Culex) pseudovishnui	6	1				3	
Culex (Culex) quinquefasciatus	3				3		1
Culex (Culex) sitiens	11					1	
Culex (Culex) tritaeniorhynchus	33		19	1	2	4	2
Culex (Lutzia) fuscanus					3	1	

*Number of collections from habitat

Table 45. Neutralization test results showing identity of 4 Wesselsbron strains

Viruses	Mouse Hyperimmune Antisera			
	BKM—367	BKM—448	BKM—589	BKM—660
			> 640	
		> 640	> 640	> 640
	10, 240	> 640	> 640	> 640
		> 640	> 640	> 640
		< 10	< 10	< 10

1/ Reciprocal of 50% plaque reduction titer.

BKM—367 was designated as the prototype strain. This agent and immune sera were sent to Dr. Jordi Casals at the Yale Arbovirus Research Unit for comparison with other group B agents. Complement fixation tests performed at YARU using BKM—367 antiserum versus 30 group B arbovirus antigens indicated a close relationship between BKM—367 virus and the H177 strain of Wesselsbron virus (table 46). Comparison of BKM—367 with Wesselsbron and West Nile viruses by cross complement fixation tests confirmed this observation (table 47).

Final confirmation of the identity of BKM—367 as a strain of Wesselsbron virus was obtained by neutralization tests which showed a close reciprocal relationship between the two viruses (table 48).

The validity of the isolations is established by the following facts:

1) No Wesselsbron or closely related viruses had ever been present in this laboratory.

Table 46. Results of complement fixation tests with BKM—367 antiserum against several Group B antigens1/.

Antigens	BKM—367 serum	Antigens	BKM—367 serum
BKM—367	128 2/	BKM—367	64
Banzi	16	Spondweni	0
Bussuquara	8	Stratford	0
Dengue 1	0	Tembusu	0
Dengue 2	8	Uganda S	8
Dengue 3	0	Usutu	8
Dengue 4	16	Wesselsbron	64
Edge Hill	16	West Nile	16
Ilheus	8	Yellow Fever	8
Turkey meningo	0	Zika	8
JBE	8	RSSE	0
Kokobera	0	OHF	0
Kunjin	8	Modoc	0
MVE	8	Rio Bravo	0
Ntaya	8	Entebbe Bat	8
SLE	16	Apoi	8
Normal tissue	0	Normal tissue	0

1/ Test performed at Yale Arbovirus Research Unit.

2/ Reciprocal of serum titer.

Table 47. Results of comparison of BKM—367, Wesselsbron and West Nile viruses by complement fixation tests.1/

Antigens	Antisera		
	BKM—367	Wesselsbron	West Nile
BKM—367	64[2/]	16	16
Wesselsbron	64	32	16
West Nile	8	8	128

1/ Test performed at Yale Arbovirus Research Unit.
2/ Reciprocal of serum titer.

Table 48. Comparison of BKM—367, Wesselsbron, and Zika viruses by neutralization tests in suckling mice*.

Antisera	Viruses		
	BKM—367	Wesselsbron	Zika
BKM—367	4.4**	3.3	
Wesselsbron	3.7	2.9	
Zika	0.1		4.4

* Tests performed at Yale Arbovirus Research Unit by Dr. Jordi Casals and Don H. Smith
** Log neutralization index

2) The agents were recovered from two mosquito species which comprised a very small percentage of the total number of mosquitoes processed for virus isolation in the laboratory during 1966.

3) Two isolations were made from each of two mosquito species and in all four instances reisolation from original mosquito pools was readily accomplished.

4) Neutralizing antibody to this agent has been found in birds and rodents collected in the same area.

Serological evidence for Wesselsbron virus (WESV) infection was obtained in a variety of vertebrates captured at Bang Phra over a two year period. As with Japanese encephalitis virus (JEV), WESV serological results present a picture of endemic maintenance involving a variety of species in all the habitat types (tables 38 and 39). Reptiles and amphibians appear to be less infected than do mammals and birds. Species resident chiefly along the bamboolined stream appear to be proportionally less infected with WESV (1/9 species positive) than with JEV (7/10 species positive). Fewer individuals were serologically WESV antibody positive than were JEV antibody positive; sera from 218 of 2,366 (9%) individuals tested neutralized WESV in the metabolic inhibition (MI) test. Fewer species were also WESV positive; sera from 37 of 107 species tested neutralized WESV as opposed to 62 of 114 which neutralized JEV.

Evidence for WESV infection of man was also obtained. A serological survey of 994 school children at Bang Phra showed age specific antibody prevalence rates of 5—50%. Although not striking, there appeared to be a gradual increase in antibody prevalence after 12 years of age (figure 19), suggesting active transmission of man over a period of at least 3 years.

The recovery of strains of Wesselsbron in Thailand is a note-worthy observation from the standpoint of arbovirus taxonomy. Previously Wesselsbron was among the so called "African Viruses" never having been found outside of the African continent. The observations reported above prove that Wesselsbron is present in S.E. Asia as well.

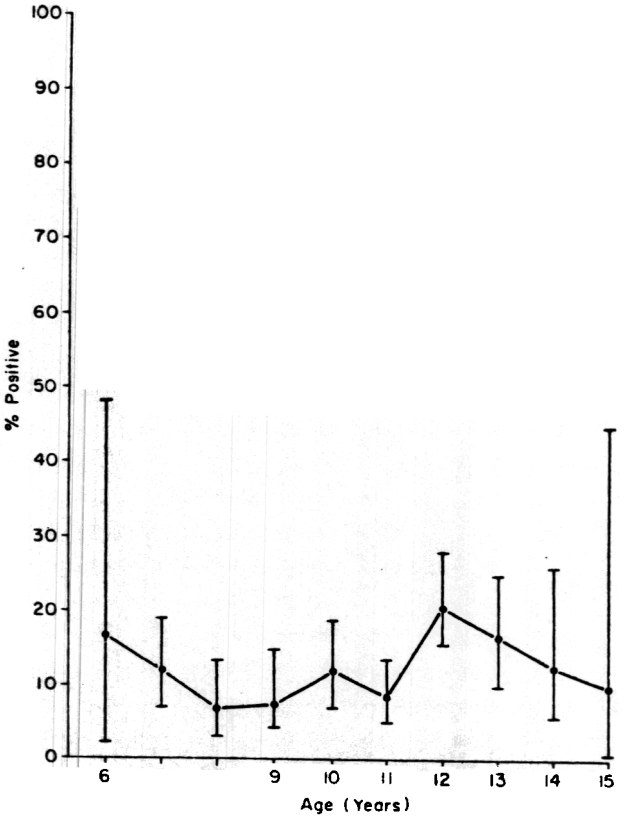

Figure 19.

African strains of Wesselsbron virus have have been shown to be capable of causing an acute febrile illness in humans. The finding of neutralizing antibody in children residing in or near Bang Phra strongly suggests that human infection with Wesselsbron virus occurs naturally in Thailand and that this agent may be a cause of febrile illness in S.E. Asia.

Recovery of Batai Virus from Aedes vexans Mosquitoes

A pool of 11 Aedes vexans female mosquitoes collected on the night of 6–7 July at Bang Phra, Thailand was found to contain a suckling mouse-lethal agent (BKM 457–66) when tested by intracerebral inoculation on 8 Aug 1966. This agent was reisolated in LLC–MK_2 cells on 15 Aug 1966 and again in suckling mice on 6 Sept 1966. Ether treatment reduced infectivity for suckling mice by $10^{3.5}$. A low-titered hemagglutinating (HA) antigen was produced with some difficulty, and reacted optimally at pH 6.2. From these characteristics, it was concluded that the agent was probably an arbovirus.

An attempt was made to place this virus into an arbovirus group. Two units of BKM 457–66 hemagglutinin were not inhibited by Group A or B antisera at 1:20 nor by specific sera to California encephalitis virus (strain BFS 283). Complement–fixation (CF) tests revealed a relationship with Bunyamwera group viruses. Grid CF tests with BKM 457–66 antigen and the homologous and Cache Valley virus (CVV) antisera resulted in antigen/antiserum titers of 128/128 and 32/16 respectively. CF titrations of other Bunyamwera group antisera against 16 units of BKM 457–66 antigen further established this group relationship (table 49).

Table 49. Complement fixation of Bunyamwera group antisera with 16 units of BKM 457—66 antigen

Anti Serum (Host)	CF Titers (Recip.) with Antigen	
	Homol.*	BKM 457—66
BKM 457—66 (Mouse)	≥256	—
Bunyamwera (Mouse)	128	64
Batai 2222 (Rab)	64	32
Germiston (Rab)	32	0
Guaroa (Mouse)	64	8
Ilesha (Mouse)	64	32
Maguari (Rab)		32
Wyeomia (Rab)	64	16

* Reciprocal dilutions of sera reacting completely with optimal concentrations of antigens.

Since Batai (MM 2222) virus is the only known Bunyamwera group virus from S.E. Asia, reciprocal cross plaque reduction neutralization tests were carried out with this virus and the Bang Phra agent. The viruses used were BKM 457—66 in the 3rd suckling mouse passage and Batai virus in the 7th suckling mouse passage. BKM 457—66 antiserum was prepared by 2 intraperitoneal injections of virus into weanling mice and the Batai antiserum was prepared by 3 intravenous injections of rabbits. Both sera neutralized BKM 457—66 virus to higher titer than Batai virus (table 50). This may have been due to presence of non infectious virus caused by the prolonged (4 years at—70°C) storage of the Batai virus seed prior to use acting as a "blocking" antigen. Despite the comparatively poorer neutralizability of Batai virus, BKM 457—66 appears to be closely related to, if not identical with it.

Table 50. Reciprocal plaque reduction neutralization tests of Batai and BKM 457—66 viruses.

Antisera		Viruses	
Designation	Host	BKM 457—66	Batai
BKM 457—66	Mouse	12,000	1,100
Batai	Rabbit	1,000	400

In the serological survey of 995 school children resident in the Bang Phra area, only two sera neutralized BKM 457—66 virus. Thus it appears that very few human infections occurred in the area where the Batai—infected mosquitoes were collected.

Summary

Experimental infections of Aedes aegypti and Aedes albopictus mosquitoes with dengue—2 virus indicated that under the conditions of the experiment both species were infected by very low doses of virus and high levels of infectious virus were present in individuals of both species by 5 days post infection.

A substance which effectively inhibits plaque production by several arboviruses was demonstrated to be present in suspensions of normal mosquitoes. Aedes aegypti and Culex tritaeniorhynchus both were shown to contain inhibitors active against dengue and JE viruses.

Comparison of several strains of JE virus from widely separated geographic regions by plaque reduction neutralization tests failed to demonstrate a significant antigenic difference between strains from mainland Asia. One strain from Sarawak, Malaysia was shown to be slightly different from other strains tested.

Detailed studies of the ecology of Japanese encephalitis in a study area in Southeast Thailand are presented. Virus transmission appeared to be continuous throughout the year at a relatively low level and involved many species of wild vertebrates. The spillover from the zoonoses to the human population resulted in low JE antibody prevalence among children in the area.

Four strains of a group B arbovirus not previously known to be present in Asia have been identified as Wesselsbron virus. Wesselsbron neutralizing antibody was found in wild vertebrates and school children in the study area.

A Bunyamwera group arbovirus recovered from Aedes vexans mosquitoes was identified as Batai virus.